Characterization of Peptide Phosphorylation Sites Using Ion Trap LC/MS/MS with Intelligent Database Searching

Application Note

Christine A. Miller and Linda L. Lopez
Agilent Technologies

Introduction

Automated, tandem mass spectrometry (MS/MS) coupled to online capillary liquid chromatography is becoming a commonly-used technique for identifying post-translational modifications of proteins. Analyses typically involve enzymatic digestion of a protein followed by capillary LC separation of the peptide fragments. As each peptide fragment elutes, intelligent, data-dependent scan functions are used to select appropriate parent ions for collision-induced dissociation (CID). Protein identification is then accomplished using probability-based computer algorithms that automatically correlate uninterpreted protein and peptide MS/MS spectra with sequences in protein or nucleotide databases. This technique not only provides the potential for automated, high-throughput data analysis, it also imparts a high degree of specificity to database searches because the tandem mass spectrum from each amino acid sequence displays a unique fingerprint-like pattern.

This note demonstrates the use of this technique for the identification of phosphorylation sites in peptides resulting from proteolytic digestion of a phosphorylated protein.

Agilent Technologies
Innovating the HP Way

Experimental

All experiments were performed using an Agilent 1100 Series LC/MSD Trap ion-trap mass spectrometer coupled to an Agilent 1100 Series Capillary LC system. The mass spectrometer was operated with the electrospray ionization (ESI) source in the positive ion mode.

Beta-casein (Sigma) was digested with trypsin (Promega) in 200 mM ammonium bicarbonate buffer for 18 hours at 37°C. The final digest had a concentration of 42 pmol/µl.

Sidebar for Figures 1, 2, and 3

ANALYSIS METHOD:

LC/MS/MS

Column:	Zorbax 300SB-C18, 150 × 0.3 mm, 5 µm
Flow rate:	5.5 µl/min
Injection volume:	100 nl
Mobile phase:	A = 0.1% formic acid in water
Mobile phase:	B = 0.1% formic acid in acetonitrile
Gradient:	5% B at 0 min 55% B at 50 min 85% B from 55–57 min
Diode-array detector:	Signal: 206, 10 nm Reference: 450, 80 nm

MS Conditions

Ionization mode:	Positive ESI
Drying gas flow:	7 l/min
Nebulizer:	15 psig
Drying gas temperature:	250°C
Skim 1:	15.0 V
Capillary exit offset:	50.0 V
Averages:	2
ICC:	On
Maximum accumulation time:	300 ms
Target:	50000
Automatic MS/MS:	Number of parents: 2 Isolation width: 3 *m/z* Fragmentation amplitude: 1.0 V

Results and Discussion

Several features of the LC/MSD Trap were used to improve the results of the data-dependent acquisition. In order to identify a maximum number of peptides, the threshold for ion current intensity was set at a level just above the chemical noise. In this way, the most intense relevant peptide ion in every MS scan automatically triggers a tandem MS experiment.

In complex chromatographic profiles with coeluting peaks, relevant peptide ions can go unanalyzed because only the ion from the more abundant of the coeluting peptides is selected for CID. To overcome this problem, the "number-of-parents" was set to two, so MS/MS spectra were acquired for the two most abundant ions from each MS scan before another MS scan was acquired. If necessary, the number-of-parents setting could have been set higher.

A 3 *m/z* isotopic-exclusion window was also used to prevent acquisition of MS/MS spectra on the ^{13}C isotopes of peptides.

Taken together, the use of data-dependent techniques including "number-of-parents" and isotopic exclusion minimized redundancy and improved data acquisition efficiency. This resulted in a significant increase in the number of unique peptide ions selected for CID, including ions of lower abundance that are often missed for MS/MS.

Figure 1 shows the chromatographic separation of a tryptic digest of bovine beta-casein by capillary LC/MS^n analysis. A total of 4 pmol was injected on column. Figure 1A shows the base peak chromatogram from LC/MS analysis of the digest. The UV absorbance was monitored at 206 nm (Figure 1B), with peptides eluting between 12 and 39 minutes. The phosphopeptide FQpSEEQQQTEDELQDK eluting at 17.5 minutes is indicated. Figures 2 and 3 show the observed full scan MS and MS/MS spectra of the doubly-charged ion at *m/z* 1032 from the bovine beta-casein phosphopeptide.

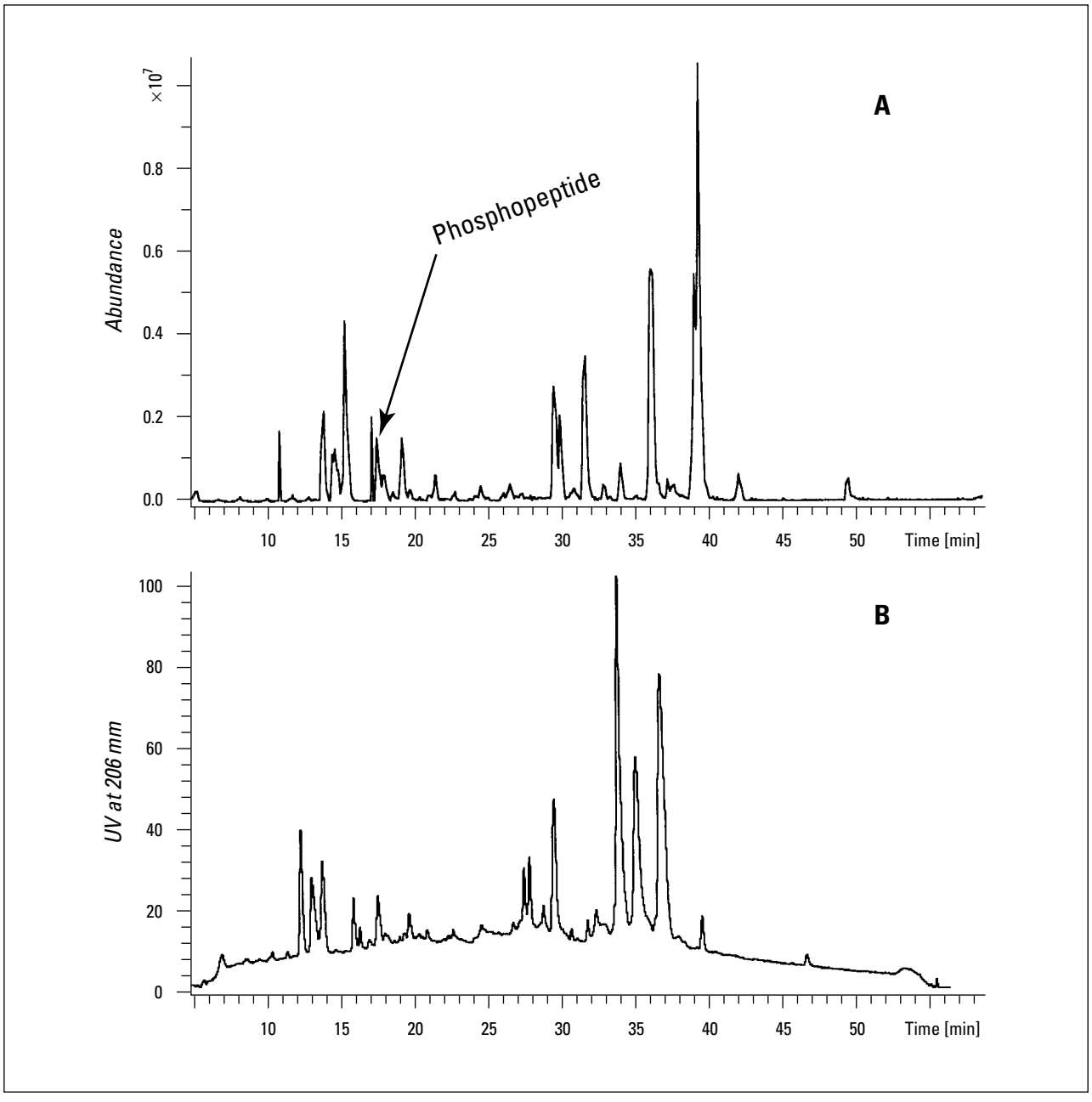

Figure 1. (A) Base peak chromatogram from capillary LC/MS^n analysis of a tryptic digest of bovine beta-casein, and (B) UV absorbance trace monitored at 206 nm.

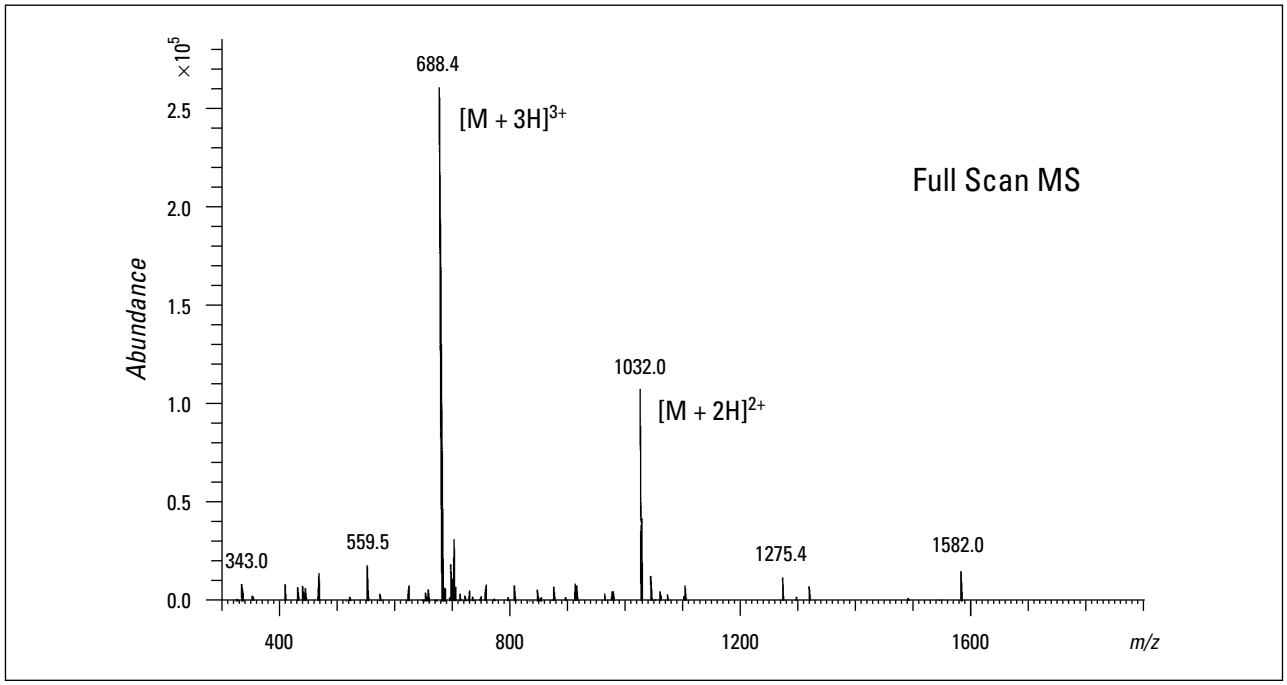

Figure 2. Full scan mass spectrum of peak at 17.5 min acquired from data-dependent analysis of a tryptic digest of bovine beta-casein.

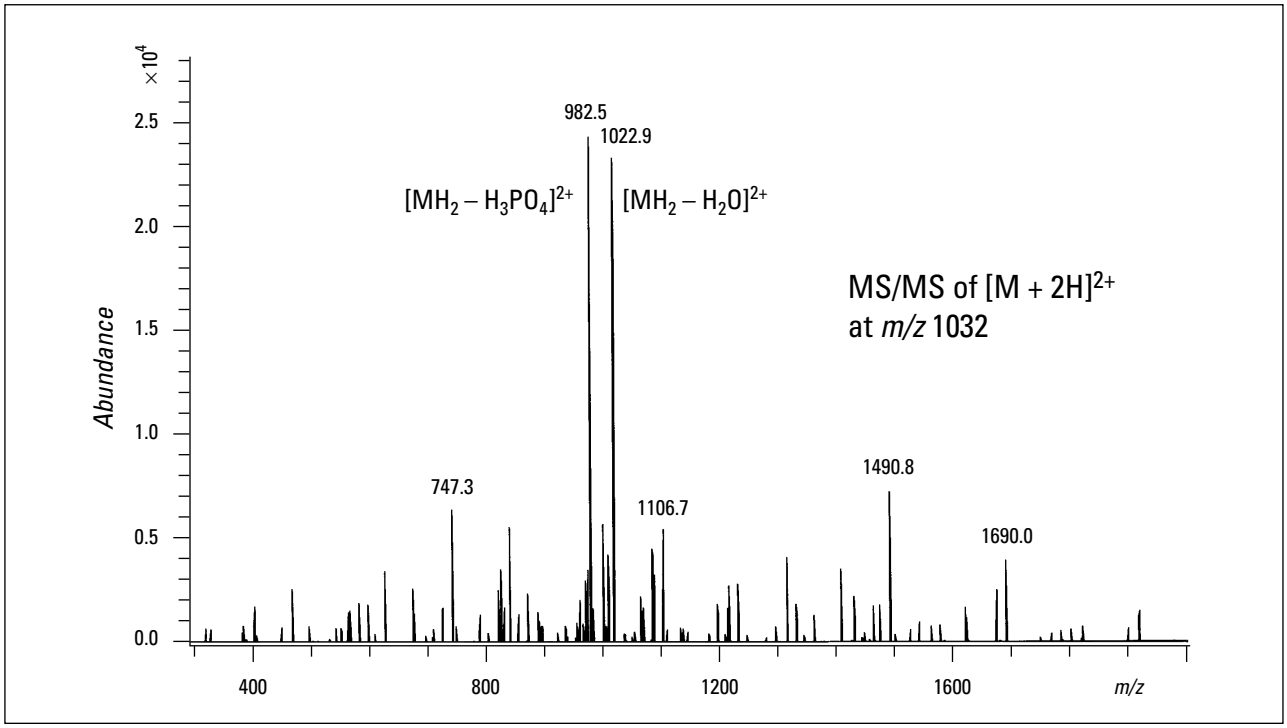

Figure 3. Full scan MS/MS spectrum of *m/z* 1032 ($[M + 2H]^{2+}$) of the peptide FQpSEEQQQTEDELQDK. The spectrum is dominated by two ions, one at *m/z* 982.5 representing the loss of H_3PO_4, and one at *m/z* 1022.9 representing the loss of H_2O.

The uninterpreted MS/MS spectra were searched using the Mascot database search program. Search parameters were set to consider possible phosphorylation at all serine, threonine, or tyrosine residues. Trypsin was specified as the enzyme. The output of the automated Mascot search gave a single, unambiguous match for the bovine beta-casein protein (Figure 4), and automatically determined a phosphorylation site on the monophosphopeptide whose sequence was confirmed to be FQpSEEQQQTEDELQDK (Table 1). The peptide results from an expected cleavage by trypsin and its observed mass of 2061.89 u is in good agreement with the calculated mass of 2061.87 u (Table 1).

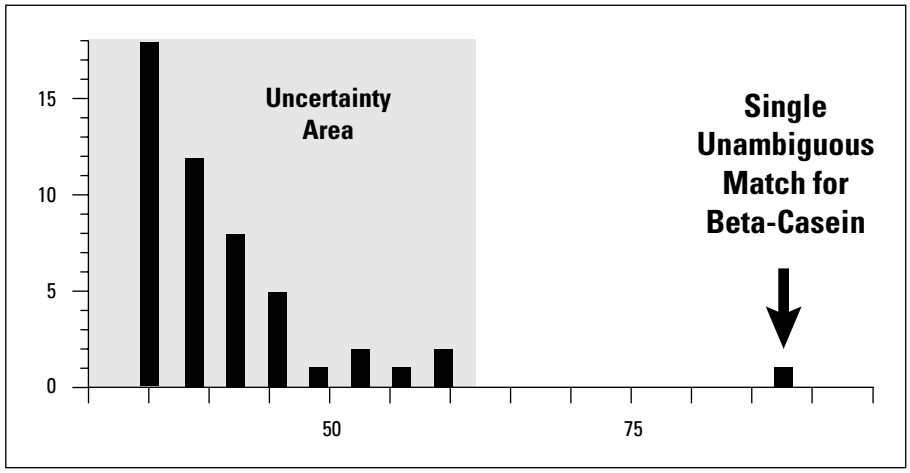

Figure 4. Results of Mascot database search showing that beta-casein is the only unambiguous match.

Table 1. Results of Mascot database search correctly identifying the phosphorylated serine residue in a phosphopeptide of bovine beta-casein.

Residues	Experimental Mass	Calculated Mass	Sequence
41 – 43	373.29	373.23	INK
44 – 47	516.49	516.33	KIEK
48 – 63	1981.29	1980.85	FQSEEQQQTEDELQDK
48 – 63	2061.89	2061.87	FQpSEEQQQTEDELQDK
113 – 120	872.69	872.48	VKEAMAPK
121 – 128	1013.09	1012.52	HKEMPFPK
192 – 198	829.69	829.44	AVPYPQR
218 – 224	741.59	741.44	GPFPIV

Conclusions

Capillary liquid chromatography coupled to electrospray ionization ion-trap mass spectrometry with intelligent database searching is a powerful tool. It enables the rapid, automated identification of peptide and protein phosphorylation sites from a single chromatographic analysis. Data-dependent MS/MS techniques increase the number of unique parent ions selected for fragmentation. Computer algorithms use this information to identify peptide and protein phosphorylation sites from uninterpreted MS/MS spectra.

Authors

Christine A. Miller and ***Linda L. Lopez*** are applications chemists at Agilent Technologies in Palo Alto, California, U.S.A.

For more information on our products and services, you can visit our site on the World Wide Web at: http://www.agilent.com/chem

Agilent Technologies shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance or use of this material.

Information, descriptions and specifications in this publication are subject to change without notice.

Copyright © 2000
Agilent Technologies
All rights reserved.
Reproduction and adaptation is prohibited.

Printed in the U.S.A. December 2000
(23) 5988-0897EN

Agilent Technologies
Innovating the HP Way